Comparative Activity of Human Carcinogens and NTP Rodent Carcinogens in the Mouse Bone Marrow Micronucleus Assay: An Integrative Approach to Genetic Toxicity Data Assessment

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The mouse bone marrow micronucleus (MN) assay holds a key position in all schemes for detecting potential human carcinogens and mutagens. It was therefore of concern when Shelby et al. reported that only 5 of 25 rodent carcinogens defined by the U.S. NTP were positive in the assay. Further, each of these positive responses was weak and indistinguishable from the 4 positive responses observed among the 24 NTP noncarcinogens tested. To focus these findings, the activity in the MN assay of 26 human carcinogens, 6 reference rodent genotoxins, and the 9 NTP chemicals positive in the MN assay have been displayed in a common format. This involved plotting the minimum positive dose level (expressed as µmole/kilogram) and the maximum fold-increase in micronucleated polychromatic erythrocytes frequency observed at any dose level. By displaying the high sensitivity of the micronucleus assay to the reference human and rodent genotoxins, this analysis emphasizes the weakness in the MN assay responses given by the NTP carcinogens reported by Shelby et al. This, in turn, poses questions about the intrinsic hazard of this selection of NTP rodent carcinogens. Using fotemustine and vitamin C as models of a toxic and a nontoxic chemical known to be active in the MN assay, this analysis describes a method by which their relative potential human hazard can be distinguished (a synthetic, as opposed to an analytical approach to data assessment). The possibility that some weak responses observed in the MN assay at elevated dose levels may be stress induced is considered. *Key words:* carcinogens, genotoxicity, mouse bone marrow micronucleus assay, mutagens, NTP. *Environ Health Perspect* 102:758–762(1994).

Over the past 5 years we have tested many human carcinogens and reference rodent genotoxins in the mouse bone marrow micronucleus (MN) assay. Without exception, activity has been observed at dose levels below the level associated with clinical signs of toxicity, and most of the responses were strong and evident at low absolute dose levels. Thus, we have become accustomed to conducting exploratory MN assays on agents suspected of having carcinogenic activity in humans at the low starting dose of 1 mg/kg. Within that context, we became concerned when Shelby et al. (*1*) reported the micronucleus results for 49 chemicals tested earlier for rodent carcinogenicity by the U.S. National Toxicology Program (NTP). Only 5 of the 25 carcinogens tested were positive, 4 of the 24 noncarcinogens tested were positive, and only 1 of these 9 positive responses exceeded by 3-fold the background micronucleated polychromatic erythrocytes (MPE) frequency (5.1-fold for monuron). Further, the toxicity of the chemicals tested by Shelby et al. (*1*) was generally low, leading to the use of dose levels that were often very high compared to those used in our studies. For example, the positive assay response reported by Shelby et al. (*1*) for vitamin C was observed at a molar dose level 1.65×10^5 higher than the dose level at which we had observed activity for the human cancer chemotherapeutic agent etoposide. We therefore decided to display and discuss our own micronucleus data, those of other investigators for the human carcinogens we have not studied, and those for the NTP chemicals found positive in the micronucleus assay by Shelby et al. (*1*).

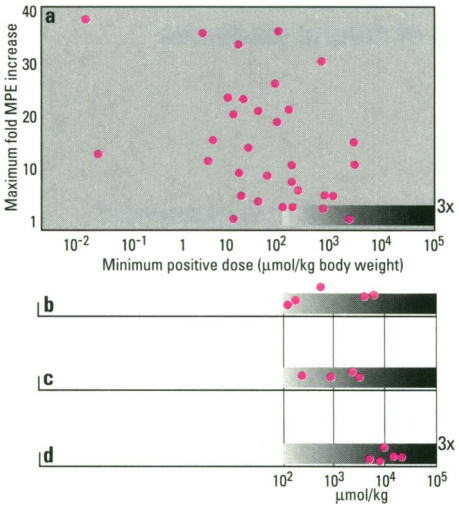

Figure 1. Maximum-fold micronucleated polychromatic erythrocyte (MPE) increase and minimum positive dose for 45 agents tested in the mouse bone marrow micronucleus assay. The high-dose/weak effect regions are shaded. (a) Twenty-six human carcinogens and 6 reference genotoxins active in the MN assay (data from Tables 1 and 2); (b) 5 of the 25 NTP carcinogens active in the MN assay (data from Table 3); (c) 4 of the 24 NTP noncarcinogens active in the MN assay (data from Table 3); (d) 5 NTP carcinogens tested at a high dose but concluded to be inactive in the MN assay (data from Table 4).

Methods

The chemicals selected for analysis and the selection criteria used are shown in Tables 1–4. The list comprises mainly agents that we have studied over the past 5 years: six widely studied rodent genotoxins, eight human carcinogens, and two suspect human carcinogens (fotemustine and etoposide). The majority of these experiments involved a single administration of the test chemical. The standard MN assay protocol used by Shelby et al. (*1*) involved three daily intraperitoneal injections of the test agent to male mice with measurements made 24 hr after the final administration. Maximum tolerated dose levels (MTD; based on observing mice for 2 days following three daily administrations) of the chemicals were administered, extending to 19,000 µmol/kg (2,500 mg/kg) for trichlorethylene (Table 4). Various adaptations of the MN assay protocol have been described, such as the use of single or repeat doses of the test agent, or use of intraperitoneal injection as opposed to oral gavage dosing (*2–5*). To date, these variables have generally led to small quantitative differences rather than qualitative changes in test outcome (*4*). We have therefore assumed that the major differences in assay responses evident between the 44 agents shown in Figure 1 must reflect differences in intrinsic genotoxicity.

Data were abstracted as the minimum positive dose and the maximum fold-increase in response reported at any dose level. Several of the chemicals shown in Tables 1 and 2 could have been tested at higher dose levels, therefore the maximum fold-increases shown sometimes represent a conservative estimate (eg., fotemustine, chlorambucil, and procarbazine). Likewise, the lowest active dose for one of the chemicals (urethane) has been estimated conservatively from strong responses seen at the only available dose levels tested. These two data parameters are plotted in Figure 1 using a unified dose scale of µmole/kilogram body weight. The chemicals are

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entered into Figure 1 in bands (A–D) to aid focused discussion. Five NTP carcinogens reported to be negative in the MN assay by Shelby et al. (*1*), but which were tested at high dose levels, and four of which gave some unreproducible evidence of activity, are also included in this analysis (Fig. 1d). Vinyl chloride is shown in Table 1 and is plotted in Figure 1 based on a conservative estimate of the dose level from the inhalation study. The oral micronucleus data for benzene (*6*) are added to Table 1 to contrast with the intraperitoneal injection study results reported by Shelby et al. (*1*).

Discussion

The established human carcinogens are, by definition, potent human carcinogens. This is because they were usually detected as the result of low-statistical-power human epidemiological studies. It is therefore interesting that most of these human carcinogens also give a strong response in the MN assay (*7–9*) (Table 1). This is most evident for the alkylating chemotherapeutic agents, but is not constrained to them, as evidenced by the potent MN assay activity of agents as structurally diverse as aflatoxin, potassium arsenite, and hexavalent chromium compounds (Table 1). The aromatic amine carcinogens benzene and nickel subsulfide give the weakest responses in the MN assay, with only phenacetin giving a weak response in terms of both dose and magnitude of effect (Table 1, Fig. 1a). The majority of the classical genotoxins (agents such as ethyl methanesulfonate, benzo[*a*]pyrene, triethylenemelamine, etc.) also give a strong response in the MN assay (*2,3*), including the six that we studied recently (Table 2, Fig. 1a). It is within the context of such carcinogen sensitivity that the mouse bone marrow MN assay was incorporated into regulatory guidelines for mutagenicity testing (*5,10*).

Only five of the 25 NTP carcinogens tested by Shelby et al. (*1*) gave a positive

Table 1. Mouse bone marrow micronucleus data for 24 human carcinogens and the 2 probable human carcinogens etoposide and fotemustine

Chemical	No. of doses	Route	Min. positive dose (μmol/kg)	Max. fold-increase	Reference
Etoposide	1	IP	0.017 (0.1 mg/kg)	37.8	(*29*)
Vincristine[a]	1	IP	0.03	14	(*30*)
N-Mustard[a]	1	IP	3.2	35.5	(*31*)
Melphalan	2	IP	4.1	13	(*32*)
Thiotepa	1	IP	5.3	16.4	(*33*)
Aflatoxins	1	PO	10	24	(*34*)
Bis(chloromethyl)ether[b]	1	IP	12.4	2.5	(*31*)
Chlorambucil	1	IP	13	21.2	(*35*)
MeCCNU	1	PO	16	33.5	(*36*)
Fotemustine	1	IP	16	10.8	(*37*)
S-Mustard	1	IP	25	15.2	(*38*)
Potassium arsenite	1	IP	38	5.9	(*39*)
Cyclophosphamide	1	PO	38	21.8	(*40*)
Treosulphan	2	IP	90	20	(*32*)
Potassium chromate	2	IP	125	4.8	(*41*)
4-Aminobiphenyl	2	IP	150	22	(*32*)
Myleran	1	IP	160	4.7	(*42*)
Azathioprine	2	PO	180	12.25	(*43*)
Chlornaphazine	1	PO	186	8.8	(*44*)
Procarbazine[a]	1	PO	226	8	(*30*)
2-Naphthylamine	1	PO	700	4.5	(*45*)
Benzidine	3	PO	810	6.9	(*45*)
Nickel subsulfide	1	IP	1040	6.8	(*46*)
Phenacetin	2	PO	2230	2.7	(*28*)
Benzene	1	PO	2820	16.5	(*6*)
Vinyl chloride		Inhalation	2820	12.5	(*47*)

Abbreviations: IP, intraperitoneal injection; PO, oral gavage.
[a]The mixture formed by N-mustard, procarbazine, and vincristine (combined chemotherapy) is carcinogenic to humans.
[b]Chloromethylether was tested because the primary human carcinogen, bis(chlormethyl)ether (BCME), is unavailable. Commercial chloromethylether contains 4% BCME. The dose-level of chloromethylether used by Morita (*31*) has been adjusted for its BCME content. The minimum positive dose level for vinyl chloride has been set conservatively at the highest dose in this table, that for benzene (*6,48*).

Table 2. Positive mouse bone marrow micronucleus assay data for six reference rodent genotoxins studied in this laboratory over the past 4 years

Chemical	No. of doses	Route	Min. positive dose (μmol/kg)	Max. fold-increase	Reference
DMH	1	PO	18	6.5	(*40*)
NOC	1	IP	20	23.8	(*30*)
DMBA	3	PO	58	10.4	(*40*)
MNU	1	PO	85	26.6	(*29*)
ENU	1	PO	97	35.9	(Tinwell, unpublished data)
Urethane	1	PO	670	30.7	(*49*)

Abbreviations: DMH, 1,2-dimethylhydrazine; NOC, nocodazole; DMBA, 7,12-dimethlybenz[*a*]anthracene; MNU, *N*-methyl-*N*-nitrosourea; ENU, *N*-ethyl-*N*-nitrosourea; IP, intraperitoneal injection; PO, oral gavage.

Table 3. Positive mouse bone marrow micronucleus data for five NTP carcinogens and four NTP noncarcinogens

Chemical	No. of doses	Route	Min. positive dose, μmol/kg	Max. fold-increase
Carcinogens				
4,4'-Methylene dianiline·2HCl	3	IP	140	1.9
4,4'-Oxydianiline	3	IP	190	2.5
Monuron (*1*)	3	IP	630	5.1
Dimethyl hydrogen phosphite	3	IP	4500	2.9
Benzene	3	IP	6400	3.0
Noncarcinogens				
2,6-Toluene diamine	3	IP	260	2.3
Phenol (*1*)	3	IP	960	2.2
Ascorbic acid	3	IP	2800	2.9
Titanium dioxide	3	IP	3100	2.4

IP, intraperitoneal injection.

Table 4. Negative mouse bone marrow micronucleus data for five NTP carcinogens[a]

Chemical	No. of doses	Route	Min. negative dose (μmol/kg)	Max. fold-increase
DEHA	3	IP	5400 (2000 mg/kg)	1.4
Benzyl acetate	3	IP	8300 (1250 mg/kg)	1.1
Cinnamyl anthranilate	3	IP	10000 (3000 mg/kg)	2.8
Melamine	3	IP	16000 (2000 mg/kg)	1.8
Trichloroethylene	3	IP	19000 (2500 mg/kg)	1.9

Abbreviations: DEHA, di(2-ethylhexyl)adipate; IP, intraperitoneal injection.
[a]These five were selected because of the elevated dose levels used and because of the weak and unreproducible assay responses for four of them.

response in the MN assay. These five positive responses were weak and observed at high dose levels (Fig. 1b). Four of the 24 NTP noncarcinogens were also active in the MN assay, showing similar levels of weak activity to those seen for the 5 NTP carcinogens (Fig. 1c). If the NTP database for the MN assay was the only one available, further use of the MN assay in screening for potential rodent carcinogens would cease.

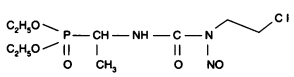
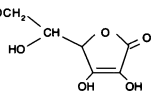
Shelby et al. (*1*) noted that several of the NTP carcinogens that were negative in the MN assay may eventually be shown to produce tumors in rodents by a nongenotoxic mechanism of action. If that were to be established, the negative MN assay responses would help define such chemicals as nongenotoxic carcinogens, rather than challenging the sensitivity of the MN assay itself. The recent suggestion by NIEHS scientists that the mouse liver carcinogen oxazepam probably produces tumors by a nongenotoxic mechanism akin to that of phenobarbital indicates that the concept of nongenotoxic carcinogenesis is gaining general credibility (*11*). The five NTP carcinogens shown in Table 4 and Figure 1d are probably representative of such nongenotoxic carcinogens. Thus, di(2-ethylhexyl)adipate and cinnamyl anthranilate induce peroxisomes in the rodent liver, and the carcinogenic status of benzyl acetate was recently reassessed when it was found to be noncarcinogenic in a repeat feed study (*12,13*). Likewise, the bladder carcinogenicity of melamine may have been caused indirectly by the induction of bladder calculi, and the mechanism(s) of action of trichloroethylene as a rodent carcinogen remains the subject of debate. The inactivity in the MN assay of such putative nongenotoxic carcinogens is therefore probably consistent. However, the possible presence of nongenotoxic carcinogens among these 25 NTP carcinogens cannot alone explain the low sensitivity of the MN assay in the Shelby et al. study. This is because no strong MN assay responses were observed in the whole study. Thus, only a fine line separates the unreproducible MN assay activities of the five carcinogens shown in Figure 1d [concluded negative by Shelby et al. (*1*)] from the weak, but positive, responses for the NTP carcinogens shown in Figure 1b. With the exception of monuron, none of these positive reponses exceeded by three times the concurrent control MPE frequencies, and all occurred at dose levels greater than 100 µmol/kg. This high-dose/weak-effect region is shown in Figure 1 and is discussed later.

There is an obvious need to understand the low carcinogen sensitivity of the MN assay in the studies reported by Shelby et al. (*1*), especially within the context of the good performance of the assay with the agents shown in Figure 1a. A speculative solution to these conflicts is shown in Figure 1 and is as follows. Certain electrophilic, or potentially electrophilic, chemicals are toxic, genotoxic, and carcinogenic to all species of animals, including humans. The most potent of these become evident as human carcinogens, usually following only limited epidemiology. Such agents are predictable as potential genotoxins from consideration of their chemical structures (*14*) and are readily detectable as rodent genotoxins (*7*) and rodent carcinogens (*15*). For such clearly genotoxic chemicals, activity in the MN assay will probably reflect an aspect of their carcinogenicity. In contrast, some chemicals are relatively nontoxic and give no obvious evidence of genotoxicity *in vivo*, or of carcinogenicity within the first year of dosing to rodents. If these chemicals are administered at the maximum tolerated dose over the lifetime of rodents, possible evidence of nongenotoxic carcinogenicity may be revealed. Some of these weaker carcinogens may also show weak activity as genotoxins *in vivo* when tested at elevated dose levels, but it is less clear if such genetic effects will relate mechanistically to their rodent carcinogenicity (Fig. 1).

The possibility that administration of chemicals to rodents at peri-toxic dose levels may induce small "stress-related" increases in MPE frequency is pertinent to this discussion. The impact of stress-related activity in the MN assay is inevitably greater for relatively nontoxic chemicals, such as vitamin C, that would normally be perceived as presenting a low hazard to humans. It is therefore interesting that the NTP carcinogens found positive in the MN assay are concentrated in the high-dose/weak-response shaded box of Figure 1. This box also contains the four false-positive MN assay responses for the NTP noncarcinogens vitamin C, titanium dioxide, phenol, and 2,6-toluenediamine. This indicates that despite the good overall correlation between carcinogenicity and activity in the MN assay, the correlation within the gray box of Figure 1 is low. This, in turn, suggests that these genetic activities might reflect a stress-induced disturbance of erythropoiesis in the test mice.

There are a few precedents for stress-induced activity in the MN assay. Water and food deprivation lead to small increases in MPE frequency in rodents (*16–18*). Similarly, increases in sister chromatid exchange levels have been observed in malnourished or pregnant humans (*19–21*). Other factors such as hyperthermia (*22,23*) and erythropoietin-induced erythropoiesis (*24,25*) have also been associated with increases in MPE frequencies in rodents. Further, methemoglobinemia induced in rodents by exposure to aniline (*26*) and spleen damage associated by their exposure to phenylhydrazine (*27*) are probably responsible for the increased incidence of MPE observed in the bone marrow of these rodents. The implication is that weak responses in the MN assay (less than threefold) observed at peri-toxic dose-levels or doses greater than approximately 100 µmol/kg should be interpreted with caution. This caution would include eight of the nine NTP positive responses reported by Shelby et al. (*1*), together with the weak positive response reported by Soutou et al. (*28*) for the human renal carcinogen phenacetin. The current problem is that there are no recognized indicators of the subtle stresses that may lead to such small increases in MPE incidences. Equally, the fact that food deprivation, for example, yields an increase in MPE incidence similar to that induced by vitamin C or titanium dioxide is a cause of recurring concern. In the absence of definitive indicators of genetic stress, the usual signs of clinical stress, such as piloerection, subdued behav-

Table 5. Illustration of the synthesis of data required to discern the differential hazard to humans of two agents active in the mouse bone marrow micronucleus (BM MN) assay (MN assay data from Tables 1 and 3)

Parameter	Fotemustine	Vitamin C
Chemical structure		
Chemical reactivity	Electrophilic	Nonelectrophilic
Metabolism	Possible formation of additional electrophiles	Anti-oxidant, radical scavenger
Salmonella assay	+	-
Mouse BM MN assay		
Qualitative response	+	+
Minimum positive dose	16 µmol/kg	2800 µmol/kg
Maximum fold-increase	11×	3×
Souce of MN assay activity	Alkylation of DNA	Radical damage to DNA following metabolic overload (?)
Carcinogenic potential to humans	+	-

ior, urinary incontinence, etc., must be used to indicate possible stress-related activity in the MN assay.

Evaluation of genetic toxicity tests has been in an analytical phase for the past 20 years. This phase was dominated by empirical validations where correlations between carcinogenicity and genetic toxicity were assessed. There has been no clear conclusion to these studies, partly because rodent carcinogenicity has fragmented into several mechanistic models during the same 20 years. In facing the contrast between the past performance of the MN assay with reference genotoxins and the human carcinogens and its performance as reported by Shelby et al. (*1*), a move away from the analytical to the synthetic/integrative approach to data assessment has been implied. This involves considering data from established genetic toxicity assays within the context of all available data for the test chemical. This includes knowledge of chemical structure, reactivity and metabolism, the general toxicology of the agent, and an appreciation of the magnitude of any induced genetic effects and the dose-levels at which they were elicited. Such a synthesis of all available data enables, for example, the potential human hazard posed by exposure to fotemustine or vitamin C to be objectively distinguished (Table 5), despite the fact that each chemical can be described as "active in the mouse bone marrow micronucleus assay." A similarly integrated approach to rodent carcinogenicity data assessment would probably also reveal a gulf between the potential carcinogenic hazard presented to humans by classical rodent carcinogens such as ethylnitrosourea and dimethylbenzanthracene (Table 2) and most of the NTP rodent carcinogens studied by Shelby et al. (*1*).

In summary, we suggest that use of the terms "rodent carcinogen" and a "micronucleus-inducing agent" in isolation of other toxicity data for the test agent should be avoided. Rather, rodent carcinogenicity and genotoxicity data should be presented and discussed within the total toxicological context available for the agent in question. Correlations between carcinogenicity and activity in the MN assay may exist in the case of agents that show strong activities for both endpoints at relatively low dose levels. However, there may be no mechanistic association between these two biological responses when they are only observed at high dose levels and when the responses are weak. Activity in the MN assay may therefore only be predictive of rodent carcinogenicity in the case of relatively strong responses (less than threefold increases over control levels) observed at low dose levels (<100 μmol/kg).

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